Factors influencing the inhibition of aflatoxin production in corn by *Aspergillus niger*[1]

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Aspergillus niger, a mold commonly associated with *Aspergillus flavus* in damaged corn, interferes with the production of aflatoxin when grown with *A. flavus* on autoclaved corn. The pH of corn-meal disks was adjusted using NaOH–HCl, citric acid – sodium citrate, or a water extract of *A. niger* fermented corn. Aflatoxin formation was completely inhibited below pH 2.8–3.0, irrespective of the system used for pH adjustment. When grown in association with *A. flavus* NRRL 6432 on autoclaved corn kernels, *A. niger* NRRL 6411 lowered substrate pH sufficiently to suppress aflatoxin production. The biodegradation of aflatoxin B_1 or its conversion to aflatoxin B_2a were eliminated as potential mechanisms by which *A. niger* reduces aflatoxin contamination. A water extract of corn kernels fermented with *A. niger* caused an additional inhibition of aflatoxin formation apart from the effects of pH.

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Aspergillus niger, une moisissure généralement associée à *Aspergillus flavus* dans le maïs endommagé, intervient dans la production d'aflatoxine lorsque cultivé avec *A. flavus* sur du maïs autoclavé. Le pH des disques de farine de maïs a été ajusté en utilisant le NaOH–HCl, l'acide citrique – citrate de sodium, ou un extrait aqueux de maïs fermenté par *A. niger*. La formation d'aflotoxine est complètement inhibée aux pH inférieurs à 2,8–3,0 peu importe le système utilisé pour ajuster le pH. Lorsque cultivé en association avec *A. flavus* NRRL 6432 sur des grains de maïs autoclavés, *A. niger* NRRL 6411 abaisse le pH du substrat suffisamment pour supprimer la production d'aflatoxine. La biodégradation de l'aflatoxine B_1 ou sa transformation en aflatoxine B_2a sont éliminées comme des mécanismes potentiels par lesquels *A. niger* réduit la contamination par l'aflatoxine. Un extrait aqueux de grains de maïs fermentés par *A. niger* cause une inhibition supplémentaire de la formation de l'aflatoxine autre que celle due aux effets du pH.

[Traduit par le journal]

Introduction

The coexistence of *Aspergillus flavus* Link ex Fr. and *Aspergillus niger* van Tiegham in cereals and other agricultural produce has been recognized for some time (Taubenhaus 1920; Semeniuk 1954; Shotwell *et al.* 1975; Manabe and Tsuruta 1978). Investigations of those variables affecting aflatoxin production by *A. flavus* have included the competitive interaction between *A. flavus* and *A. niger*. Ashworth *et al.* (1965) found that aflatoxin did not develop when *A. flavus* and *A. niger* were co-inoculated onto autoclaved peanuts. Tsubouchi *et al.* (1981) also reported substantial to total inhibition of aflatoxin production when autoclaved rice was simultaneously inoculated with *A. flavus* and *A. niger*. Similarly, *A. niger* completely inhibited aflatoxin formation on autoclaved corn when paired with *A. flavus* (Wicklow *et al.* 1980). In the latter example, *Aspergillus niger* did not exclude *A. flavus* from the corn, but appeared to share or subdivide the resource in that each fungus sporulated in patchy sectors over an equivalent area of the kernel surface. The inhibition of aflatoxin production attributed to *A. niger* may therefore involve factors apart from the competitive exclusion of *A. flavus* from the substrate.

The objective of this study is to attempt explanation of the mechanism by which *A. niger* reduces aflatoxin contamination when simultaneously cultured with *A. flavus*. The following hypotheses are examined: (*i*) *Aspergillus niger* degrades aflatoxin; (*ii*) the pH of the substrate is lowered sufficiently by *A. niger* to inhibit aflatoxin formation; and (*iii*) a metabolite(s) of *A. niger*, or a compound(s) produced through the modification of the substrate on which *A. niger* grows, interferes with aflatoxin biosynthesis.

Methods

Fungus inoculum

The two mold strains used in these experiments, *Aspergillus flavus* NRRL 6432 and *A. niger* NRRL 6411, were isolated from aflatoxin-contaminated corn sampled at harvest in 1977 from fields in North Carolina.

Cell and conidial suspensions (propagule density = 1×10^6/mL) of each fungal isolate, prepared in 0.01% Triton-X-100 from 14-day-old cultures on Czapek's agar, represented the inoculum. For co-inoculations of *A. flavus* and *A. niger*, equal volumes of each suspension were combined before inoculation.

Extract of A. niger *fermented corn*

To prepare cultures for the water extract of *A. niger* fermented corn, 100 g of whole corn kernels was placed in

[1]The mention of firm names or trade products does not imply that they are endorsed by the U.S. Department of Agriculture over other firms or similar products not mentioned.

each of four Fernbach flasks (2800 mL), soaked 24 h in 500 mL of distilled water at 25°C, and steam sterilized. Free water was decanted. The kernels were inoculated with a cell and conidial suspension of *A. niger* NRRL 6411 and incubated for 14 days at 28°C. Following incubation, the four Fernbach cultures were combined and extracted twice with 1 L of distilled water in a Waring blendor. The extracts were then combined and evaporated to 350 mL with a flash evaporator (Buchler Instruments, Fort Lee, NJ). A portion of the final extract (1 mL) was plated on potato dextrose agar to ensure no viable propagules of *A. niger* were present. The extract was stored at 5°C and used within 1 week.

Solid substrate preparation

Whole corn kernels and corn-meal disks served as solid substrates for the fermentations. The whole corn kernels were first soaked for 24 h in distilled water at 25°C. A small wound was then made in the germ of each kernel, and 10 of these kernels were autoclaved (germ side up) in a glass petri dish containing two moistened No. 1 Whatman filter papers. One drop (0.05 mL) of a conidial suspension was inoculated onto the wound of each kernel. The kernels were incubated for 7 days at 28°C in the dark.

Corn-meal disks were used in some experiments to effectively adjust the pH of the solid substrate. The corn-meal disks were prepared by mixing 20 g corn meal (Quaker; yellow, enriched) with either 20 mL distilled water or an equal volume of 0.5 *N* citric acid – sodium citrate buffer or water extract of *A. niger* fermented corn. To adjust substrate pH, the citrate buffer and water extract were adjusted to pH prior to addition to the corn meal; adjustments of pH with 3 *N* NaOH–HCl were made after the corn meal had been mixed with water. The corn meal was then spread evenly in a 15 × 100 mm glass petri dish, cooked briefly by autoclaving (5 min), and cooled. Ten disks were removed from the corn meal using a No. 7 cork borer (14 mm diameter) and steam sterilized in a glass petri dish with moistened filter papers. The inoculation and incubation of the corn-meal disks were the same as for the whole corn kernels. Quantities of aflatoxin produced by *A. flavus* NRRL 6432 were considerably less on the corn-meal disks than on whole kernels, possibly owing to differences in physical and (or) nutritional factors (i.e., the corn meal was degerminated). However, the variability within treatments was low, thus allowing an accurate measurement of the effects of the variables being tested.

Measurement of substrate pH

For each treatment, two plates containing whole corn kernels or corn-meal disks were included in addition to those strictly for aflatoxin analysis: one plate to measure pH after sterilization but prior to inoculation; the other plate for pH measurement after 7 days of incubation with *A. flavus* and (or) *A. niger*. Kernels or disks from each plate were macerated in 20 mL distilled water to determine pH.

Degradation of aflatoxin

The ability of *A. niger* to degrade aflatoxin was determined by injecting individual corn kernels with 2.5 μg aflatoxin B_1 (5 μL standard containing 0.5 μg/μL aflatoxin B_1 in acetonitrile–benzene (2:98, v/v). Following injection, kernels were autoclaved and then inoculated with *A. niger* NRRL 6411. Aflatoxin levels were assayed after 0, 3, 5, 7, and 9 days of incubation.

Aflatoxin analysis

Test plates for each experimental treatment were prepared and used in triplicate, with each plate consisting of 10 individual corn kernels or corn-meal disks. Following fermentation, the contents of each plate were extracted with 100 mL dichloromethane–methanol (70:30, v/v) for 2 min in a Waring blendor. The extract was then filtered through Whatman 2V filter paper, evaporated to dryness with a flash evaporator, and reconstituted in 10 mL dichloromethane. Aflatoxin concentration was determined by thin-layer chromatography using plates precoated with 0.25-mm silica gel (MCB Manufacturing Chemists, Inc., Cincinnati, OH). Plates were developed in chloroform–acetone (85:15, v/v, plus 0.25% distilled water) and the aflatoxin B_1 was quantified using a spectrodensitometer (Schoeffel Instrument-Corp., model SD3000) at a wavelength of 365 nm and an emission of 445 nm. Aflatoxin values obtained from kernels spiked with a known quantity of aflatoxin B_1, and subsequently incubated with *A. niger* NRRL 6411, were determined using thin-layer chromatography and visually comparing extract spots with those of a series of aflatoxin standards. The identity of aflatoxin B_1 was confirmed by the formation of the water adduct, aflatoxin B_2a (Anonymous 1980).

Data on the effect of *A. niger* NRRL 6411 on aflatoxin B_1 production by different strains of *A. flavus* were examined as a 2 × 4 factoral experiment using a three-way analysis of variance ($P = 0.05$) with a log transformation of the aflatoxin values. For data from other experiments, one-way analyses of variances ($P = 0.05$) were calculated using a log transformation of the aflatoxin values.

Results and Discussion

When *A. flavus* strains of different aflatoxin-producing capabilities were paired with *A. niger* NRRL 6411 on autoclaved corn kernels, levels of detectable aflatoxin B_1 were significantly reduced ($P < 0.05$) as contrasted with *A. flavus* inoculated controls (Table 1). Aflatoxin production was not completely inhibited by *A. niger* on autoclaved corn as previously reported by Wicklow *et al.* (1980). Even so, the decrease in aflatoxin B_1 ranged from 70% (NRRL 3357) to 96% (NRRL 6555). With all strains of *A. flavus* tested, conidial sporulation of *A. flavus* and *A. niger* on kernel surfaces occupied sectors of approximately equal area (Fig. 1). Since *A. flavus* and *A. niger* sporulate equally on kernel surfaces, the decrease in aflatoxin B_1 would be greater than expected owing solely to the competitive exclusion of *A. flavus* from the substrate.

To determine the mechanism by which *A. niger* reduces aflatoxin contamination below levels that might be predicted solely from the effects of competitive exclusion, one of our strains, *A. flavus* NRRL 6432, was chosen for further investigation. Our first approach was to examine the possibility that *A. niger* degrades aflatoxin. Of the 25 μg aflatoxin B_1 added to the 10 kernels in each test plate, approximately 44% was recoverable from the 0-day controls (Fig. 2). A one-way analysis of variance indicates that a linear trend in the aflatoxin values was not significant ($P > 0.05$) following

TABLE 1. Effect of *Aspergillus niger* NRRL 6411 on aflatoxin B_1 production by different strains of *Aspergillus flavus* grown on autoclaved corn kernels

A. flavus strain	Final pH[a,b]	Aflatoxin B_1 (ppb)[b] Mean	Aflatoxin B_1 (ppb)[b] Range	% decrease in aflatoxin B_1
NRRL 3357	4.9	91 900	45 100 – 118 000	
3357 + *A. niger*	3.1	27 100	25 000 – 30 800	70%
NRRL 6432	5.0	25 600	23 400 – 28 300	
6432 + *A. niger*	3.7	2 750	1 540 – 4 800	89%
NRRL 6555	5.2	14 300	10 000 – 16 900	
6555 + *A. niger*	3.2	579	356 – 953	96%
NRRL 6412	5.0	10 800	4 920 – 16 200	
6412 + *A. niger*	3.2	1 360	782 – 2 020	87%
A. niger	2.8	—	—	
Uninoculated (control)	6.2	—	—	

[a]Initial pH = 6.2.
[b]After 7 days incubation at 25°C.

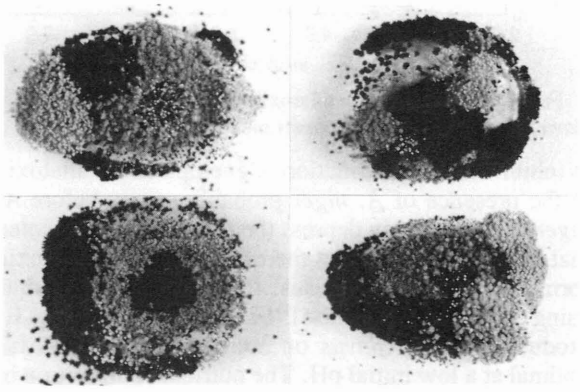

FIG. 1. Autoclaved corn kernels co-inoculated with *Aspergillus flavus* NRRL 6432 and *Aspergillus niger* NRRL 6411. Black sectors represent conidial heads of *A. niger*; other mold growth represents yellow–green conidial heads of *A. flavus*. Incubation was for 7 days at 25°C.

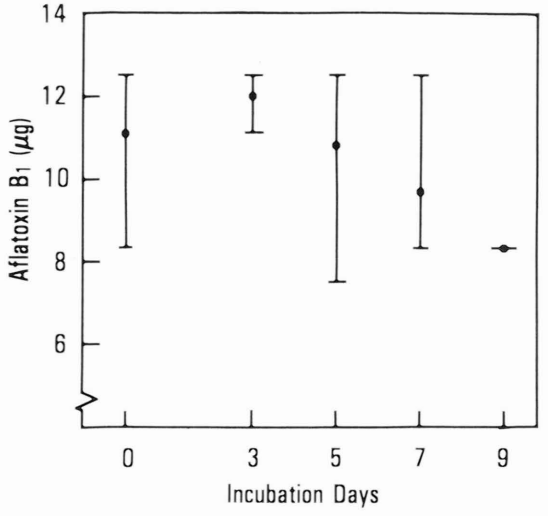

FIG. 2. Amounts of aflatoxin recovered from autoclaved corn kernels spiked with aflatoxin B_1 (25 μg/10 kernels) following incubation with *Aspergillus niger* NRRL 6411 at 25°C. Aflatoxin values represent the mean of three replicates per treatment; ranges are indicated by lines.

0, 3, 5, 7, and 9 days incubation with *A. niger*. Although we did not detect significant degradation of aflatoxin by *A. niger*, other investigators have shown that in liquid culture *A. niger* can effectively degrade aflatoxin B_1 (Ashworth *et al.* 1965; Mann and Rehm 1977; Tsubouchi *et al.* 1980). *Aspergillus niger* is also capable of nonenzymatically converting aflatoxin B_1 to a related compound, aflatoxin B_2a, by lowering the pH of the medium (Ciegler *et al.* 1966; Ciegler and Peterson 1968). In our fermentations, we were unable to detect aflatoxin B_2a. Although significant degradation or conversion of aflatoxin B_1 may have occurred upon further incubation of our kernels, other inhibitory effects of *A. niger* on aflatoxin production appear to be active in our fermentations.

Because strains of *A. niger* are capable of producing large quantities of various organic acids (Raper and Fennell 1965), we investigated the possibility that by reducing the pH of the substrate, *A. niger* inhibits the formation of aflatoxin. Our results show that aflatoxin production by *A. flavus* on corn-meal disks was significantly reduced ($P < 0.05$) at lower pH values, irrespective of whether the pH adjustment was with 3 *N* NaOH–HCl, 0.5 *N* citric acid – sodium citrate, or the water extract of *A. niger* fermented corn (Tables 2 and 3). Aflatoxin B_2a was not detected in any of these corn-meal disks nor was the degree of conidial sporulation affected by pH except at the lowest pH values of the

TABLE 2. Effect of pH on aflatoxin production by *Aspergillus flavus* NRRL 6432 when grown on corn-meal disks

Initial pH	Final pH[a]	Aflatoxin B_1 (ppb)[a,b] Mean	Range
3.2	3.8	32	22–44
3.6	3.9	90	60–122
5.4	4.9	320	70–359
6.7	5.2	318	21–449
7.2	5.6	627	96–820
6.0 (Control)	5.3	1390	860–1880
pH adjustment with 0.5 *N* citric acid – sodium citrate			
2.8	2.9	ND	
3.4	3.1	ND	
4.5	4.6	762	613–919
5.4	5.3	1930	1590–2430
6.2	5.5	1150	331–1770
5.9 (Control)	5.0	1060	735–1390

[a]After 7 days incubation at 25°C.
[b]ND, not detected.

TABLE 3. Effect of *Aspergillus niger* NRRL 6411 water extract at different pH levels on aflatoxin production by *Aspergillus flavus* NRRL 6432 when grown on corn-meal disks

Initial pH[a]	Final pH[b]	Aflatoxin B_1 (ppb)[b,c] Mean	Range
2.4	2.5	ND[d]	
2.7	3.8	ND[e]	
4.4	5.2	19	ND–32
4.9 (Unadjusted)	5.4	66	20–154
5.0	5.7	142	115–158
5.7	5.8	102	69–149
6.2	6.2	163	127–189
6.6	6.4	193	192–194
6.0 (Control, water)	5.3	1270	671–1990
6.0 (Uninoculated)	6.0	—	—
6.0 (*A. niger*)	3.0	—	—

[a]pH adjustment with 3 and 5 *N* NaOH–HCl.
[b]After 7 days of incubation at 25°C.
[c]ND, not detected.
[d]No visible sporulation of *A. flavus*.
[e]Minimal sporulation of *A. flavus*.

water extract. In Fig. 3, a least-squares line of the data points for each experiment reveals that aflatoxin becomes undetectable below pH 2.8–3.0. When cultured alone on whole corn kernels or corn-meal disks, *A. niger* NRRL 6411 lowered substrate pH from an initial 5.9–6.2 to 2.8–3.0 after 7 days of incubation. In contrast, isolates of *A. flavus* depressed the pH to only 4.9–5.3. When *A. niger* was paired with different strains of *A. flavus* on autoclaved corn kernels, the substrate pH was lowered from 6.2 to 3.1–3.7 (Table 1). These data indicate that *A. niger*, when associated with *A. flavus*, may reduce the pH of the substrate sufficiently

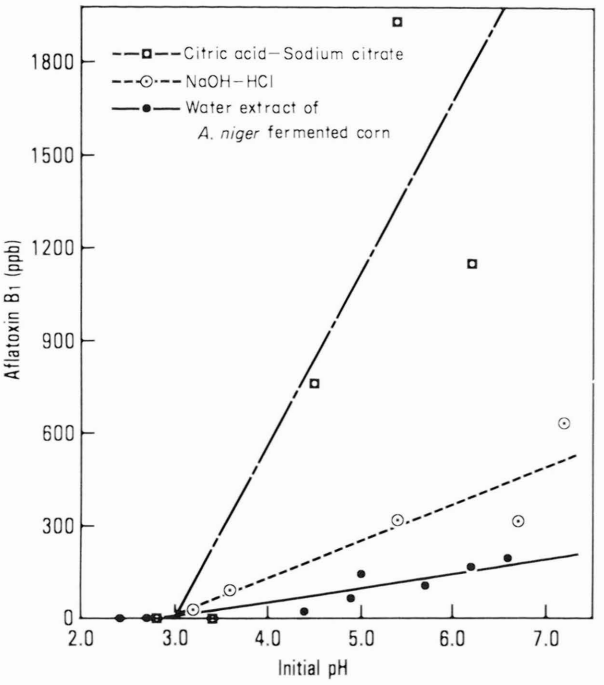

FIG. 3. Effect of pH on aflatoxin production by *Aspergillus flavus* NRRL 6432 when grown on corn-meal disks.

to inhibit aflatoxin formation. Development of aflatoxin in the presence of *A. niger* probably occurs before *A. niger* can effectively depress the pH. It should be noted that little is known about the effect of pH on aflatoxin formation in solid substrates. In contrast to our results using corn, Lie and Marth (1968) found that aflatoxin B_1 production by *A. flavus* on cottage cheese curd was optimal at a low initial pH. The nutrient composition of solid substrates may be a factor in determining the effect of pH on aflatoxin production, as has been shown to be the case with liquid culture media (Buchanan and Ayres 1975).

Our data indicate that at a given pH aflatoxin values can differ considerably, depending upon how the pH is adjusted (Fig. 3). In the 3 *N* NaOH–HCl treatments, a significant reduction ($P < 0.05$) in aflatoxin production at higher pH values was observed compared with the water control at pH 6.0 (Table 2). This effect can possibly be attributed to a higher solute concentration resulting from the adjustment of pH. Uraih and Chipley (1976) reported that a high salt concentration may adversely affect aflatoxin production by *A. flavus*. Aflatoxin synthesis by *A. parasiticus* can also be inhibited by suboptimal water activity, but without a marked inhibition of mycelial growth (Northolt *et al.* 1976). At pH 4.5 and above, the 0.5 *N* citric acid – sodium citrate buffer had no significant effect ($P > 0.05$) on aflatoxin production compared with the water control (Table 2). Equilibria would favor citrate ion at these pH values. According to Hendricks (1964), undissociated organic carboxylic acids (such as citric acid) rather than

their ionized states (sodium citrate) tend to be transported through cell membranes where they can retard (or enhance) cell metabolism. Citric acid can also have the indirect effect of chelating metals such as zinc (Martell and Calvin 1952), which is required for aflatoxin production (Mateles and Adye 1965; Gupta and Venkitasubramanian 1975). Although aflatoxin production was inhibited at lower pH values where citric acid concentrations are higher relative to sodium citrate, the response was similar in three different adjustments of pH. It therefore appears that the inhibition is probably an effect of pH rather than a response to citric acid.

The water extract of *A. niger* fermented corn showed an additional inhibition of aflatoxin production apart from the effects of pH (Fig. 3). This inhibition can possibly be attributed to the high solute concentration of the extract, which could retard growth of *A. flavus* and (or) aflatoxin production. However, the role of a chemical factor(s) produced by *A. niger* and inhibitory to aflatoxin biosynthesis cannot be discounted.

Our research using autoclaved corn kernels and corn-meal disks as an experimental system to study the effects of *A. niger* on aflatoxin production by *A. flavus* indicates the following: (*i*) aflatoxin is not degraded by *A. niger*; (*ii*) substrate pH is lowered sufficiently by *A. niger* to inhibit aflatoxin formation; (*iii*) an additional inhibition of aflatoxin formation is exhibited by the water extract of *A. niger* fermented corn. In naturally contaminated stored corn, the effect of variables such as substrate moisture, temperature, extent of kernel damage, amount of inoculum, and order of inoculation on *A. flavus* – *A. niger* interaction may account for the reports that *A. niger* has little or no effect on aflatoxin formation (Fennell *et al.* 1973; Bothast *et al.* 1976; Seitz *et al.* 1982). Studies are currently underway to assess the effect of *A. niger* on aflatoxin formation by *A. flavus* when co-inoculated onto preharvest corn.

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